Cyclosporine A normalizes mitochondrial coupling, reactive oxygen species production, and inflammation and partially restores skeletal muscle maximal oxidative capacity in experimental aortic cross-clamping

Julien Pottecher, MD, PhD,[a,b] Max Guillot, MD,[b,c] Elise Belaidi, PhD,[d] Anne-Laure Charles, PhD,[b,e] Anne Lejay, MD,[b,f] Abdallah Gharib, PhD,[d] Pierre Diemunsch, MD, PhD,[a,b] and Bernard Geny, MD, PhD,[b,e] *Strasbourg and Lyon, France*

Objective: By binding to cyclophilin D, cyclosporine A (CsA) inhibits mitochondrial permeability transition pore (mPTP) opening and prevents mitochondrial dysfunction and ultimately cell death after ischemia-reperfusion (IR) injury in cardiac muscle. This study tested whether CsA would decrease skeletal muscle oxidative stress and mitochondrial dysfunctions after aortic cross-clamping related IR.

Methods: Forty-five Wistar rats were investigated. The sham group (n = 8) had aortic exposure but no ischemia, the IR group (n = 10) had aortic cross-clamping for 3 hours followed by 2 hours of reperfusion, and the IR+CsA group (n = 9) had two intraperitoneal injections of 10 mg of CsA at 90 and 150 minutes of ischemia before reperfusion. Mitochondrial coupling (acceptor control ratio) and mitochondrial respiratory chain complexes' activities were measured. Reactive oxygen species (ROS) production, cyclophilin D expression, and muscle inflammation were determined using dihydroethidium staining, Western blot, and immunohistochemistry, respectively. An additional 18 sham rats were investigated to determine CsA blood levels and the effects of CsA on mitochondrial respiration and calcium retention capacity, a marker of mPTP opening, both in myocardium and gastrocnemius with and without CsA.

Results: Compared with sham, IR decreased mitochondrial coupling (1.38 ± 0.06 vs 1.98 ± 0.20; $P = .0092$), increased ROS production (3992 ± 706 arbitrary units [AU] vs 1812 ± 322 AU; $P = .033$), was associated with macrophage infiltration, and decreased maximal oxidative capacity (V_{max}: 4.08 ± 0.38 μmol O_2/min/g vs 5.98 ± 0.56 μmol O_2/min/g; $P = .015$). Despite IR, CsA treatment totally restored mitochondrial coupling (1.93 ± 0.12; $P = .023$ vs IR), normalized ROS (1569 ± 348 AU; $P = .0098$ vs IR), and decreased inflammation. The V_{max} was slightly enhanced (5.02 ± 0.39 μmol O_2/min/g; $P = .33$ vs IR; $P = .35$ vs sham). Compared with myocardium, gastrocnemius muscle was characterized by a decreased cyclophilin D content (−50%) associated with an earlier opening of mPTP (calcium retention capacity increased from 10.85 ± 1.35 μM/mg dry weight [DW] to 12.11 ± 2.77 μM/mg DW; $P = .65$; and from 11.07 ± 1.67 to 37.65 ± 11.41 μM/mg DW; $P = .0098$ in gastrocnemius and heart, respectively).

Conclusions: Cyclosporine A normalized ROS production, decreased inflammation, and restored mitochondrial coupling during aortic cross-clamping. Incomplete V_{max} protection might be due to low cyclophilin D expression in gastrocnemius, preventing CsA from blocking mPTP opening. (J Vasc Surg 2013;57:1100-8.)

Clinical Relevance: Aortic cross-clamping increases reactive oxygen species production and impairs skeletal muscle mitochondrial function. Even subtle muscle impairments may increase morbidity after abdominal aortic aneurysm repair, supporting a need for muscle protection during vascular surgery. Albeit its protective effects might be related to muscle content in cyclophilin D, this study demonstrates that cyclosporine A significantly alleviates ischemia-reperfusion injury in skeletal muscle. Because cyclosporine A can be used safely in humans, it might be an alternative or a synergistic therapeutic approach to ischemic preconditioning in order to reduce mitochondrial dysfunction and reactive oxygen species production in vascular patients undergoing aortic cross-clamping.

From Pôle Anesthésie Réanimation Chirurgicale, SAMU, Hôpitaux Universitaires de Strasbourg,[a] Equipe d'Accueil 3072, Institut de Physiologie, Faculté de Médecine, Université de Strasbourg,[b] and Service de Réanimation Médicale, Hôpital de Hautepierre,[c] Strasbourg; Inserm U1060-CarMen & service d'Explorations Fonctionnelles Cardiovasculaires, Hospices Civils de Lyon & Université Claude Bernard Lyon1, Lyon[d]; and Service de Physiologie et d'Explorations Fonctionnelles, Pôle de Pathologie Thoracique, Hôpitaux Universitaires de Strasbourg,[e] and Service de Chirurgie Cardiovasculaire, Pôle de Cardiologie, Hôpitaux Universitaires, CHRU de Strasbourg,[f] Strasbourg.

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Reprint requests: Professeur Bernard Geny, Service de Physiologie et d'Explorations Fonctionnelles et Equipe d'Accueil 3072, Université et Hôpitaux Universitaires de Strasbourg, 67091 Strasbourg, France (e-mail: bernard.geny@chru-strasbourg.fr).

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Open abdominal aortic aneurysm repair is a surgical intervention commonly carried out in high-risk surgical patients with advanced cardiovascular disease and complicated outcomes.[1] It requires aortic cross-clamping, which induces skeletal muscle ischemia-reperfusion (IR) injury that largely participates in perioperative and long-term morbidity.[2,3] Several mechanisms are involved in these alterations, with key factors being increased reactive oxygen species (ROS) production and impaired mitochondrial function. Particularly, the mitochondrion is a central player in cell survival. Indeed, mitochondria are the source of adenosine triphosphate (ATP) storage, the main core of ROS generation[4] and the mainstay of apoptosis regulation. At the same time, the mitochondria are an early target of IR mechanisms. Indeed, ROS overproduction mediates IR-induced mitochondrial dysfunction in skeletal muscles.[5] Molecular pathways then converge to a mitochondrial key target, the mitochondrial permeability transition pore (mPTP), whose opening causes both cell necrosis and apoptosis.[6]

In order to rescue skeletal muscle from infarction, local and remote ischemic preconditioning equivalently protected muscles' mitochondrial function against IR-induced injury.[7] Ischemic postconditioning, characterized by repeated cycles of IR performed at the onset of reperfusion, was also thought to be protective, but recent data suggest it should be used with caution.[8,9] Furthermore, repetitive vascular clamping in atherosclerotic patients is neither always feasible nor devoid of side effects,[10] so there is obviously a need for an alternative approach.

Pharmacologic conditioning is an option, and cyclosporine A (CsA), a potent mPTP opening inhibitor, is extremely powerful in protecting cardiomyocytes from IR.[11] Indeed, CsA binds to cyclophilin D and inhibits mPTP opening, preventing mitochondrial dysfunction, oxidative phosphorylation uncoupling, and ultimately cell death.[12] Cyclophilins are ubiquitous proteins, highly conserved during evolution and identified in the genomes of mammals, plants, insect, fungi, and bacteria.[13] This suggests that CsA effects should also be observed in a variety of tissues, including human tissues. Other intracellular mechanisms also could be at play. Thus, reduced ROS production and reduced inflammation potentially might participate in CsA biologic effects.[14] Recently, Askar and Bozkurt[15] administered CsA before femoral occlusion and observed decreased numbers of rolling, sticking, and transmigrating neutrophils in skeletal muscles submitted to 4 hours of ischemia and 24 hours of reperfusion. Importantly, acute CsA administration should not be associated with deleterious effects linked to persistent immunosuppression. Thus, short-term CsA does not affect cutaneous or musculofascial wound healing in clinically relevant dose.[16]

The aim of this study was to test the hypothesis that, by preventing mPTP opening, CsA would preserve mitochondrial coupling and mitochondrial complex activities, and decrease ROS production and inflammation in gastrocnemius muscles submitted to IR. To obtain further insight into the mechanisms involved in CsA effects, we also investigated whether cyclophilin D expression and mPTP opening might differ in myocardium and gastrocnemius muscles.

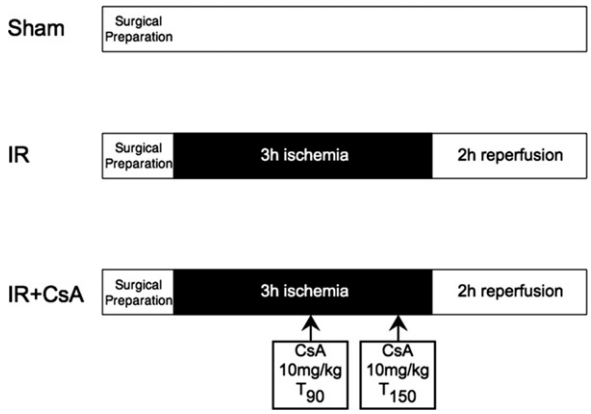

Fig 1. Experimental design. Sham animals (*Sham*; n = 8) underwent 5 hours of general anesthesia and were sham operated. Ischemia-reperfusion animals (*IR*; n = 10) underwent 3 hours of ischemia induced by infrarenal aortic occlusion, followed by 2 hours of reperfusion. Cyclosporine-conditioned rats (*IR+CsA*; n = 9) also underwent 3 hours of ischemia, but complete reperfusion was preceded by two intraperitoneal cyclosporine A (*CsA*) injections (10 mg/kg each) at 90 and 150 minutes of ischemia.

METHODS

Procedures were conducted in accordance with U.S. National Institutes of Health guidelines.

Animals. Experiments were performed on adult male Wistar rats (Depré, Saint Doulchard, France) weighing 250 to 300 g. Animals were housed in a neutral temperature environment (22° ± 2°C) on a 12:12 hour photoperiod and were provided food and water ad libitum.

Preoperative management. Rats were anesthetized with a gas mixture containing 4% isoflurane and oxygen, in a hermetic anesthetic induction cage (Aerrane; CSP, Cournon, France). Spontaneous ventilation was allowed through an oxygen-delivering mask, with different concentrations of isoflurane depending on the surgical phase (2% during painful stimuli and 1% during latent periods). The rat's temperature was maintained constant at 37°C using blanket control (homeothermic blanket control unit; MINERVE, Esternay, France) and monitored with rectal probe.

Surgical procedure and experimental design. Twenty-seven rats were divided into three groups (Fig 1). The sham group (n = 8) underwent 5 hours of isoflurane anesthesia. A midline laparotomy was performed. The posterior peritoneum was cut to expose the abdominal aorta and the inferior vena cava. Infrarenal abdominal aorta was dissected and liberated from adjacent adhesions in all animals.

The IR group (n = 10) underwent 3 hours of ischemia induced by infrarenal aortic occlusion and collateral vessels ligation, followed by 2 hours of reperfusion. The aorta was temporally occluded using a DeBakey cross-action bulldog clamp, and all arterial collaterals located between the renal

arteries and the aortic bifurcation were coagulated and cut using a thermal cautery (Geiger thermal cautery unit; Geiger Medical Technologies, Council Bluffs, Iowa), as previously reported.[7,8] After aortic clamping, the abdomen was tightly closed to prevent dehydration. Ischemia was clinically characterized by cyanosis and lack of arterial pulse distal to the clamp. It was also ascertained by capillary lactate measurements on the right foot before unclamping (Lactate Pro device, LT1710; Arkray, KGK, Kyoto, Japan).[17] Lactates are end products of cellular anaerobic energetic metabolism, a key biomarker of tissue hypoxia. Using the same aortic clamping model, we observed increased lactates and decreased skeletal muscle mitochondrial function, objectifying the degree of limb ischemia.[7,8]

The CsA-conditioned rats (IR+CsA; n = 9) also underwent 3 hours of ischemia, but complete reperfusion was preceded by two intraperitoneal 10 mg/kg CsA injections (Sandimmun; Novartis-Pharma SAS, Rueil-Malmaison, France) at 90 and 150 minutes of ischemia. Dose, administration route, and timing were selected based on CsA pharmacokinetic properties and protective doses in rats.[18,19] Translated to IR protocols, an optimal CsA level was expected at the time of reperfusion, albeit a further increase might occur until the end of the experiment.[18,20]

We additionally included 18 sham rats (seven treated with CsA, 11 untreated) to determine the effects of the same CsA injections on mitochondrial respiration and calcium retention capacity (CRC), a marker of mPTP opening. In the two groups, CRC was assessed in both myocardium and gastrocnemius muscles, and mitochondrial respiration was determined in gastrocnemius muscle.

Blood CsA assay. Blood samples (500 μL) from additional CsA-treated sham rats were drawn from the internal jugular vein on ethylenediaminetetraacetic acid tubes after 3 hours of general anesthesia when blood CsA near peaked and analyzed using the MassTox reagent kit (Chromsystems, Munich, Germany) as described in the Appendix (online only).

Tissue processing. At the end of the experiment, white superficial gastrocnemius muscles were excised and cleaned of adipose and connective tissue. One part was kept on ice and immediately used for studying mitochondrial respiration in skinned fibers. Delay between excision and beginning of analysis remained <20 minutes, thus avoiding secondary ischemia. The second part was immediately frozen in isopentane cooled by liquid nitrogen and stored for subsequent analysis.

Study of muscle mitochondrial respiration in skinned fibers. Mitochondrial respiration was studied in saponin-skinned fibers, as previously described.[9] Measuring oxygen consumption ex vivo in skinned fibers is a unique way to determine the functional oxidative capacity of skeletal muscle, allowing the study of the entire mitochondrial population within its cellular environment.

Briefly, fibers were separated, then permeabilized, in a bath of solution containing 50 μg/mL saponin for 30 minutes at 4°C, under shaking. The permeabilized fibers then were washed for 10 minutes under shaking in order to remove saponin. Fibers were placed in a bath with the respiratory solution for 5 minutes twice in order to remove any phosphates. Finally, oxygen consumption was measured polarographically using a Clark-type electrode in a 3-mL oxygraphic cell (Strathkelvin Instruments, Glasgow, Scotland).

Basal oxygen consumption (V_0), and maximal fiber respiration (V_{max}) rates were measured at 22.1°C under continuous stirring in the presence of saturating amount of adenosine diphosphate (ADP) as a phosphate acceptor. Relative contributions of the respiratory chain complexes I, III, and IV to the global mitochondrial respiratory rates also were determined. When V_{max} was recorded, electron flow went through complexes I, III, and IV. Complex I was blocked with amobarbital (Amytal; 0.02 mM), and complex II was stimulated with succinate (25 mM). Mitochondrial respiration then allowed determination of complex II, III, and IV activities (V_{succ}). N, N, N′, N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5 mM) and ascorbate (0.5 mM) were added as artificial electron donors to cytochrome c. Under these conditions, the activity of cytochrome c oxydase (complex IV) was determined as an isolated step of the respiratory chain ($V_{TMPD/Asc}$). Mitochondrial coupling (coupling of phosphorylation to oxidation) was determined by calculating the acceptor control ratio (ACR), the ratio between ADP-stimulated respiration (V_{max}) over basal respiration (without ADP) with glutamate and malate as substrate (V_0). Fibers were then harvested and dried for 15 minutes at 150°C. Respiration rates are expressed as μmol O_2/min/g dry weight (DW).

Dihydroethidium staining. To detect the presence of ROS in skeletal muscles, serial sections (10 μm thick) were cut on a cryostat microtome and incubated with dihydroethidium (DHE), which produces red fluorescence when oxidized to ethidium bromide by ROS including superoxide anion (see Appendix, online only).

Immunohistochemical procedure. To detect gastrocnemius inflammation, we used monocyte macrophage-2 (Millipore, Billerica, Mass) antibody on 10-μm-thick serial sections of muscles (see Appendix, online only).[9]

Study of cyclophilin D expression in gastrocnemius and myocardium. Cyclophilin D expression (relative to β-actin) was measured by Western blot (Appendix, online only)

Calcium retention capacity measurements in gastrocnemius and myocardium. The resistance of mPTP to opening after matrix Ca^{2+} challenge was determined in permeabilized "ghost" muscle fibers, characterized by a lack of myosin, allowing Ca^{2+} uptake only by mitochondria and prepared as described in the Appendix (online only). The amount of Ca^{2+} necessary to trigger a massive Ca^{2+} release was used as an indicator of the susceptibility of mPTP to Ca^{2+} overload and expressed as μM/mg DW. The Ca^{2+} concentration was calculated from a standard curve relating [Ca^{2+}] to the fluorescence of calcium green.

Statistical analysis. All data are expressed as mean ± standard error of the mean and analyzed using the Prism database (GraphPad Prism 5; Graph Pad Software, San

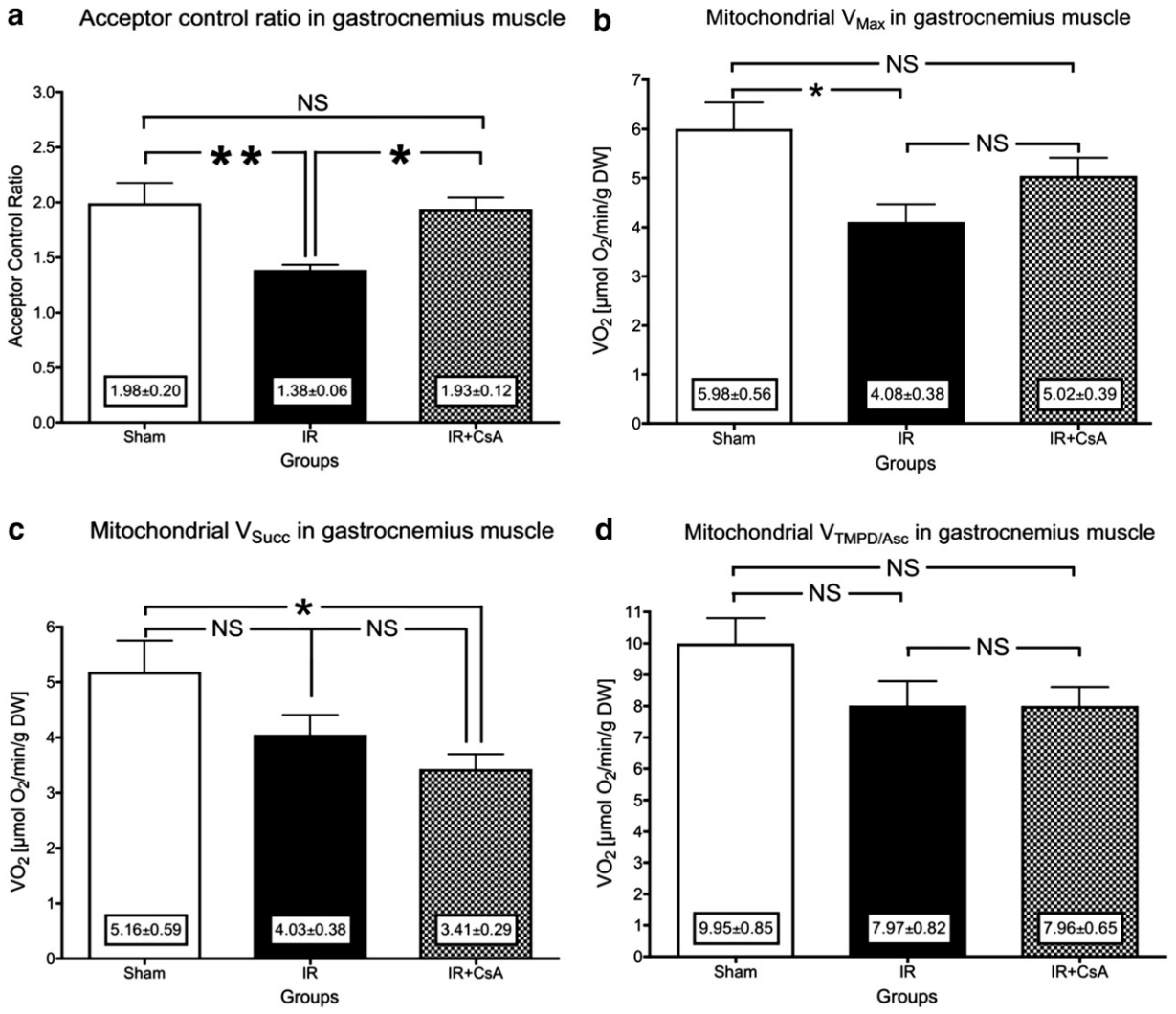

Fig 2. Effects of ischemia-reperfusion (*IR*) and cyclosporine A (*IR+CsA*) on mitochondrial coupling and respiratory chain complexes activities. **a,** Acceptor control ratio (ACR). **b,** Maximal fiber mitochondrial respiration rates, complexes I, III, and IV (V_{max}). **c,** Combined activity of mitochondrial complexes II, III, and IV (V_{succ}). **d,** Complex IV activity ($V_{TMPD/Asc}$). *NS,* Not significant. $*P < .05$; $**P < .01$.

Diego, Calif). One-way analysis of variance was used in all groups, with post-test analysis between groups using the Newman-Keuls test correcting for multiple comparisons. $P < .05$ (two-tailed) was considered significant. As previously observed, eight to 10 rats per group were required to demonstrate a 30% reduction in mitochondrial function after IR.[7-9]

RESULTS

Effects of aortic cross-clamping and reperfusion on skeletal muscle mitochondrial function, ROS production, inflammation, and cyclophilin D content. As expected, capillary lactate measurements revealed that aortic cross-clamping significantly increased anaerobic metabolism in the IR group compared with the sham group (16.6 ± 1.0 mmol/L vs 2.7 ± 0.4 mmol/L, respectively; $P < .0001$).

IR decreased muscle mitochondrial coupling and respiratory chain complexes activities. The ACR was significantly reduced (from 1.98 ± 0.20 to 1.38 ± 0.06; −30%; $P = .0092$; Fig 2, *a*). The V_0 tended to decrease after IR from 3.16 ± 0.34 to 2.96 ± 0.28 µmol O_2/min/g DW in sham and IR, respectively. The V_{max} was significantly decreased (from 5.98 ± 0.56 µmol O_2/min/g DW to 4.08 ± 0.38 µmol O_2/min/g DW; 32%, $P = .015$; Fig 2, *b*) after 3 hours ischemia and 2 hours reperfusion in gastrocnemius muscle. V_{succ} and $V_{TMPD/Asc}$ decreases were not statistically significant after IR (Fig 2, *c* and *d*).

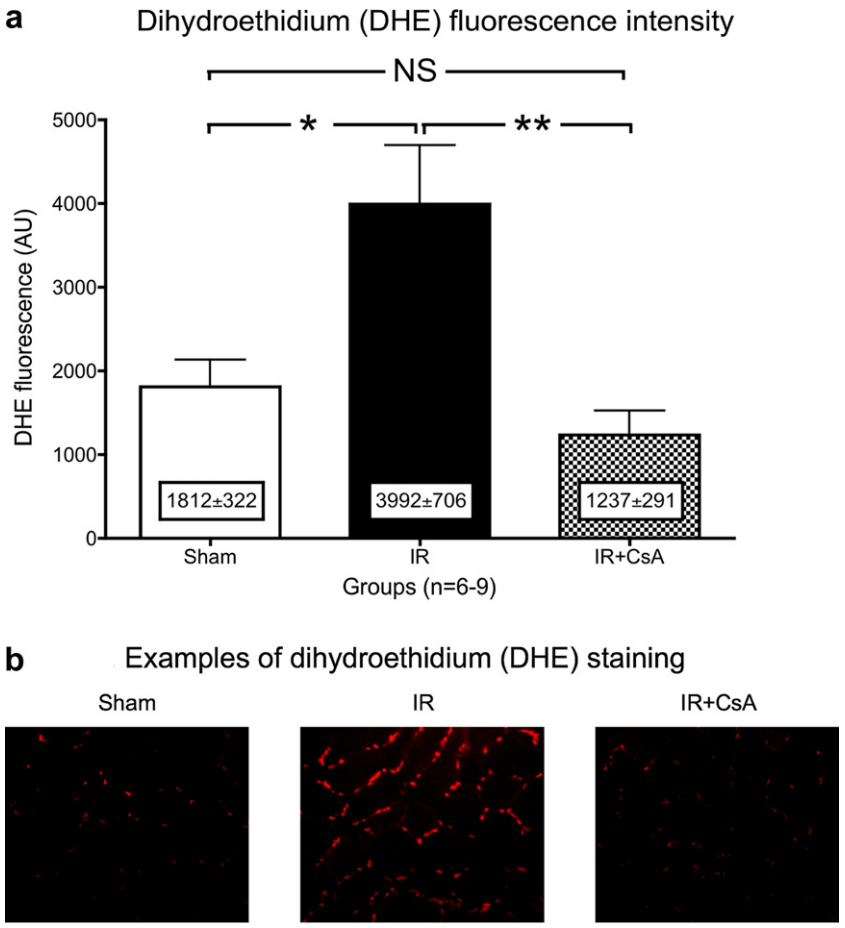

Fig 3. Skeletal muscle production of reactive oxygen species in sham, after ischemia-reperfusion (*IR*), and after cyclosporine A (*IR+CsA*). **a,** Dihydroethidium (*DHE*) fluorescence intensity (arbitrary units [*AU*]). **b,** Examples of DHE staining. *NS,* Not significant. **P* < .05; ***P* < .01.

IR increased muscle ROS production and inflammation. Hind limb IR led to a significant increase in DHE fluorescence in gastrocnemius sections (from 1812 ± 322 arbitrary units [AU] to 3992 ± 706 AU; +120%; $P = .033$), reflecting an increase in ROS, including superoxide anion generation (Fig 3). Similarly, IR increased macrophage infiltration (Fig 4).

IR did not significantly affect muscle cyclophilin D expression. Cyclophilin D expression (relative to β-actin) in gastrocnemius muscle was not significantly different in IR vs sham animals (1.29 ± 0.14 vs 1.63 ± 0.24; $P = .29$).

Effects of CsA in sham rats and on IR-induced deleterious effects

Effects of CsA on normal gastrocnemius muscles. In the additional CsA-treated sham rats, mitochondrial respiratory chain complex activities were not significantly different from their respective values in untreated sham animals (Table, online only).

Cyclosporine A preserved mitochondrial respiratory chain coupling and partly protected complex I activity against IR. Acknowledging that CsA absorption might be modulated by surgical exposure and peritoneal inflammation, in sham, blood CsA concentration was 2420 ± 147 μg/L after 3 hours of general anesthesia. This likely allowed potential biologic effects, as inferred from previous data in the cardiac IR setting.[20]

Accordingly, CsA conditioning significantly improved the ACR (from 1.38 ± 0.06 to 1.93 ± 0.12; $P = .023$ vs IR) and restored its value close to the nonischemic control (1.98 ± 0.20; $P = .95$ vs sham; Fig 2, *a*). Indeed, V_0 tended to be reduced (2.69 ± 0.25 μmol O_2/min/g DW), and V_{max} (5.02 ± 0.39 μmol O_2/min/g DW) tended to be increased compared with the IR group (4.08 ± 0.38 μmolO_2/min/g DW; $P = .33$ vs IR; Fig 2, *b*).

Concerning V_{succ}, IR alone induced a nonsignificant decrease. Combining IR and CsA rendered the V_{succ} decrease significant compared with control (Fig 2, *c*). However, V_{succ} was similar when comparing IR and IR+CsA, thus supporting that the decreased activity in

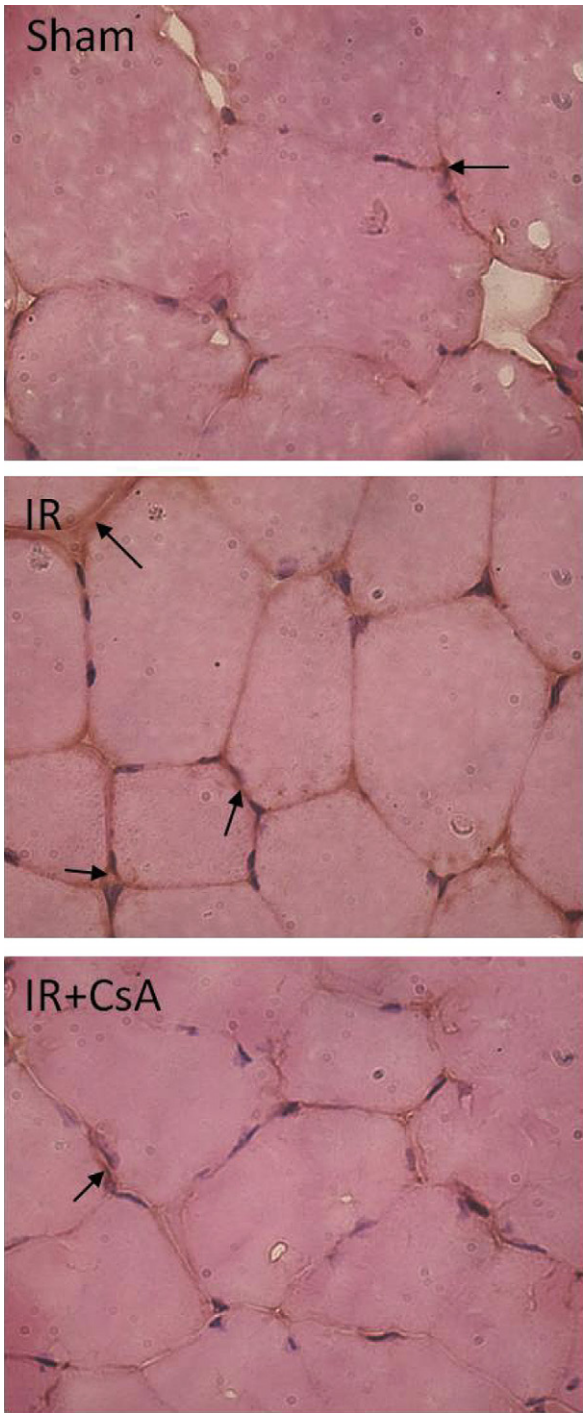

Fig 4. Effects of ischemia-reperfusion (*IR*) and cyclosporine A (*IR+CsA*) on inflammation. Immunohistochemical demonstration of the distribution of macrophages. *Black arrows* indicate the presence of macrophages (original magnification, ×400).

complexes II, III, and/or complex IV after IR+CsA was mainly due to IR.

Similarly, CsA conditioning alone did not induce a significant change in $V_{TMPD/Asc}$ compared with IR (Fig 2, *d*).

Taken together, because CsA did not increase complex II, III, and IV activities, the beneficial effects observed for V_{max} should be due mainly to an effect on complex I.

Cyclosporine A reduced muscle ROS production and inflammation. The CsA conditioning decreased DHE fluorescence in gastrocnemius sections (from 3992 ± 706 AU to 1569 ± 348 AU; $P = .0098$; Fig 3), indicating decreased generation of ROS and therefore decreased oxidative stress in skeletal muscles submitted to IR. Macrophage infiltration was also reduced by CsA administration (Fig 4).

Mechanisms of CsA effects: implication of cyclophilin D content and CRC

Cyclophilin D expression in heart and gastrocnemius muscles. Cyclophilin D expression relative to β-actin was twice as large in myocardium compared with gastrocnemius in sham rats (5.52 ± 0.86 AU vs 2.19 ± 0.38 AU; $P = .009$; Fig 5, *a*).

Functional implication of reduced cyclophilin D content in gastrocnemius: CsA reduces CRC in heart but not in gastrocnemius muscle. To investigate whether the decreased level of cyclophilin D expression in gastrocnemius was functional, we tested the resistance of mPTP to opening after matrix Ca^{2+} challenge in heart and gastrocnemius with or without CsA. As expected, the Ca^{2+} load required to open the mPTP was significantly increased by CsA treatment in myocardium (from 11.07 ± 1.67 μM Ca^{2+}/mg DW to 37.65 ± 11.41 μM Ca^{2+}/mg DW; $P = .0098$). Conversely, the Ca^{2+} load required to open the gastrocnemius mPTP was not different with and without CsA (from 10.85 ± 1.35 μM Ca^{2+}/mg DW to 12.11 ± 2.77 μM Ca^{2+}/mg DW; $P = .65$; Fig 5, *b* and *c*), supporting that CsA did not modify gastrocnemius mPTP sensitivity to calcium load.

DISCUSSION

This study confirms that IR impairs skeletal muscle mitochondrial coupling and respiratory complex activities and increases oxidative stress. Furthermore, after aortic cross-clamping, CsA normalizes mitochondrial coupling, ROS production, and macrophage infiltration. CsA also partially restores mitochondrial respiratory chain complex activities, likely in relation with gastrocnemius cyclophilin D content.

Human skeletal muscle withstands 2.5 hours of warm global ischemia before histologic injuries appear[21] and muscular ATP pool is exhausted.[22] However, in surgical clinical practice, although 3 hours of aortic clamping time is longer than in most surgical procedures, aortic clamp may be prolonged in robot-assisted laparoscopy and/or by unpredictable complications such as arterial thrombosis and vasospasm. Knowing that even subtle muscle impairments may increase morbidity after abdominal aortic aneurysm repair,[2] muscle protection during vascular surgery appears mandatory.

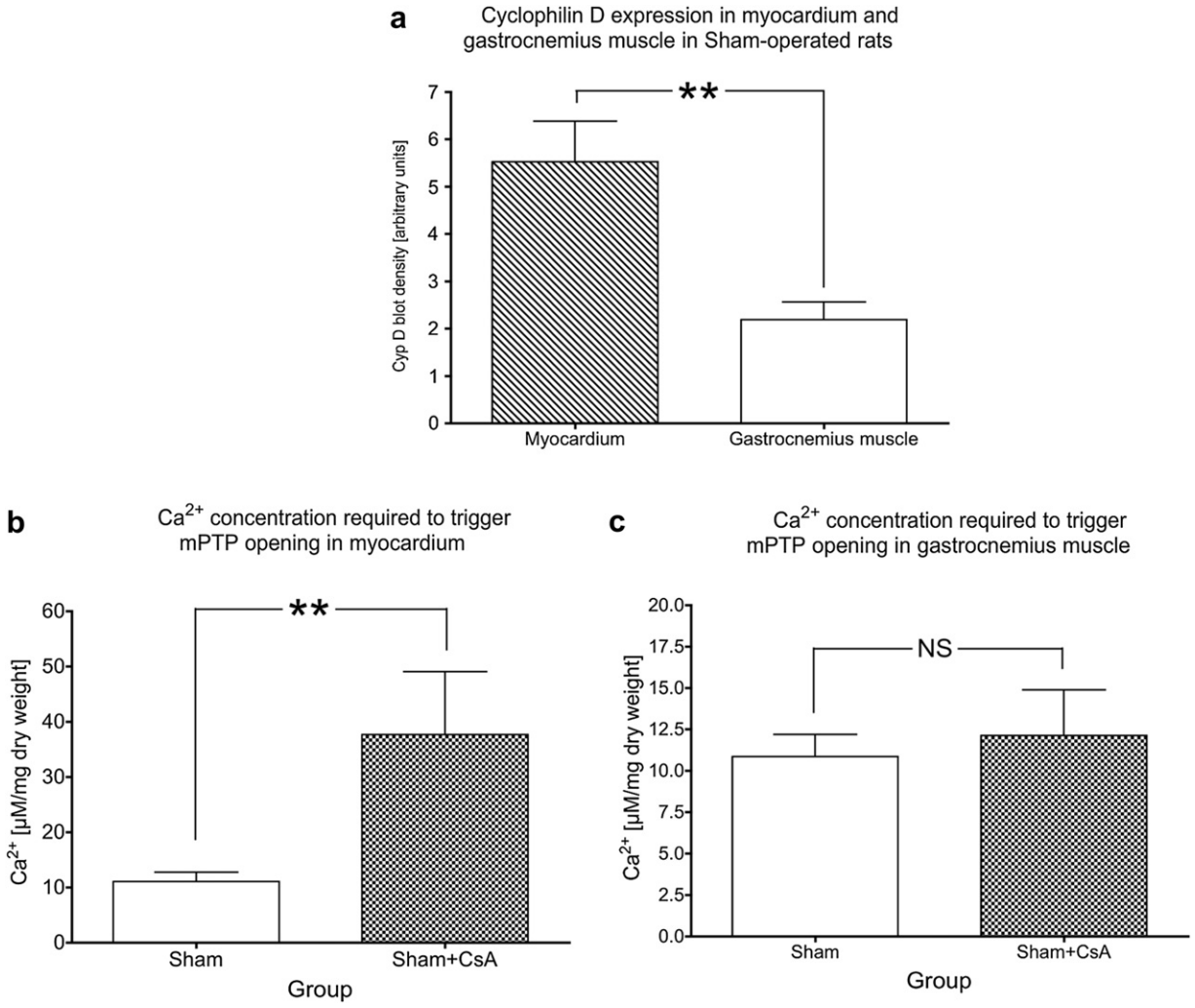

Fig 5. Cyclophilin D expression and Ca^{2+} concentration required to trigger mitochondrial permeability transition pore (*mPTP*) opening in sham heart and gastrocnemius muscles. **a,** Cyclophilin D expression in myocardium and gastrocnemius muscles. **b,** Ca^{2+} concentration required to trigger mPTP opening in myocardium. **c,** Ca^{2+} concentration required to trigger mPTP opening in gastrocnemius muscle. *CsA*, Cyclosporine A; *NS*, not significant. $^{**}P < .01$.

Aortic cross-clamping significantly impaired both mitochondrial coupling and mitochondrial oxidative capacity, supporting that mitochondrial respiratory chain dysfunction is a sensitive marker of IR injury. Hind limb IR also increased ROS production, as inferred from the enhanced DHE staining in IR muscle. Similarly, IR increased muscle inflammation. These data, in line with previous results,[5,8] further support that mitochondrial dysfunction and increased oxidative stress and inflammation are key factors involved in IR mechanisms.

Several therapies, including controlled reperfusion[23,24] and ischemic preconditioning[7,25] and postconditioning,[8,26] have been shown effective in protecting hind limbs against IR-deleterious effects. Sometimes difficult to apply, such approaches might be associated with or perhaps replaced by easier to perform pharmacologic approaches. Thus, there is a general agreement that pharmacologic conditioning with CsA is effective in protecting the myocardium from IR injury.[11,20]

In our study, CsA totally restored mitochondrial coupling. Accordingly, rats fed with CsA 10 mg/kg/d and 25 mg/kg/d for 3 weeks significantly decreased their gastrocnemius V_0 (−40% and −45%, respectively), and CsA dose dependently increased the ACR in gastrocnemius muscles.[27] Changes in V_0 were less marked here, perhaps because of the shorter duration of the experiments, but our results are in line with these data.[27]

Reduced oxidative stress likely participated in CsA beneficial effects, as other protective approaches such as ischemic postconditioning also decrease IR-induced ROS production and muscle mitochondrial dysfunction.[5,8] Accordingly, a constant cross-talk takes place between the coupling of phosphorylation to oxidation and mitochondrial ROS generation.[28] Mitochondrial

dysfunction and ROS generation feed off each other, perpetuating a vicious circle that eventually leads to cell death. As the mitochondrial respiratory chain is disrupted, coupling decreases and more electrons leak; these are involved in the generation of ROS leading to oxidative stress and mPTP opening. In particular, mitochondrial complexes I and III are the main sources of ROS production during IR. However, when glutamate and malate are used as mitochondrial substrates, the major site of ROS production is complex I through reverse flux of electrons from complex II to complex I.[29] If CsA preserves complex I function, it prevents further ROS release from this mitochondrial complex. Accordingly, CsA clearly reduced ROS production. This is consistent with a previous study demonstrating that CsA improved ATP generation and decreased muscle myeloperoxidase activity after 4 hours of ischemia and 2 hours of reperfusion.[15] This fact, along with mitochondrial properties of skeletal muscle being preserved in heart transplant recipients, suggests that CsA was not harmful.[30]

Finally, there are interactions between CsA and inflammation in skeletal muscle. The CsA has been shown to decrease muscular neutrophil infiltration during IR.[15] Accordingly, CsA reduced macrophage infiltration in our study.

Interestingly, however, despite its beneficial effects on mitochondrial coupling and ROS production, CsA only partially protected skeletal muscle maximal oxidative capacity. Several factors might limit CsA efficiency.

CsA vehicle can specifically decrease complex I activity without altering mitochondrial coupling.[31] Complex I activity of the mitochondrial respiratory chain largely participates in V_{max}, and incomplete restoration of V_{max} might partly be explained by a potential deleterious effect of CsA vehicle on complex I. Nevertheless, such data were obtained in vitro using a concentration range much higher than that used in our study. Compared with peak CsA values observed 3 hours after administration in late heart transplant recipients (808 ± 295 ng/mL), our blood CsA concentrations appeared higher.[32] However, they were well within the effective ranges previously demonstrated to protect myocardium from IR injury.[20] Furthermore, our additional experiment showed that similar CsA doses did not affect gastrocnemius mitochondrial function. Thus, an alternative explanation should be explored.

CsA cardioprotection is obtained through its binding and inhibition of cyclophilin D, a mitochondrial protein favoring mPTP opening and therefore apoptosis. It is conceivable that CsA protection depends on cyclophilin D content in muscle cells.[33,34] To further investigate the mechanisms of CsA protective effects, we determined the expression of cyclophilin D in both skeletal and cardiac muscles and measured their CRC, a marker of mPTP opening. Gastrocnemius cyclophilin D expression was decreased compared with myocardium. This was associated with functional changes. Indeed, CsA significantly delayed myocardial mPTP opening in response to Ca^{2+} load but failed to delay gastrocnemius mPTP opening. Accordingly, the inhibitory effect of CsA on mPTP opening was greater in case of increased cyclophilin D content in denervated muscle.[33] Thus, CsA protection might depend on cyclophilin D expression in muscle, possibly explaining CsA prevention of infarction flaps in pig latissimus dorsi muscle characterized by a high level of cyclophilin D expression.[26,35]

Another potential mechanism explaining our results may be inferred from the actions of CsA upon B- and T-cell activation. The CsA upregulates immunoglobulin production in preactivated B cells,[36] but CsA inhibits calcineurin-dependent nuclear factor of activated T cell dephosphorylation and interrupts the transcription of genes coding for interleukin-2, interleukin-4. and CD40 ligand. This decreases the efficacy of T-cell–dependent immune defenses[37] and deserves further study.

CONCLUSIONS

The CsA normalized ROS production during aortic cross-clamping and restored mitochondrial coupling. Incomplete V_{max} protection might be due to low cyclophilin D expression in gastrocnemius, preventing CsA from blocking mPTP opening. Although controversial, combining CsA and ischemic conditioning might be a promising alternative because they can have additive effects.[38-40] This new therapeutic approach might warrant being proposed to patients undergoing prolonged vascular surgery procedures in order to reduce skeletal muscle impairment and therefore patient morbidity.

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AUTHOR CONTRIBUTIONS

Conception and design: JP, MG, BG
Analysis and interpretation: JP, MG, EB, AC, AL, AG, PD, BG
Data collection: JP, MG, EB, AC, AL
Writing the article: JP, AG, BG
Critical revision of the article: JP, MG, EB, AC, AL, AG, PD, BG
Final approval of the article: JP, MG, EB, AC, AL, AG, PD, BG
Statistical analysis: JP
Obtained funding: BG
Overall responsibility: JP, BG

REFERENCES

1. Lovegrove RE, Javid M, Magee TR, Galland RB. A meta-analysis of 21,178 patients undergoing open or endovascular repair of abdominal aortic aneurysm. Br J Surg 2008;95:677-84.
2. Ali ZA, Callaghan CJ, Lim E, Ali AA, Reza Nouraei SA, Akthar AM, et al. Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. Circulation 2007;116:I98-105.

3. Miller CC III, Villa MA, Sutton J, Lau D, Keyhani K, Estrera AL, et al. Serum myoglobin and renal morbidity and mortality following thoracic and thoraco-abdominal aortic repair: does rhabdomyolysis play a role? Eur J Vasc Endovasc Surg 2009;37:388-94.
4. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. Biochim Biophys Acta 2006;1757: 509-17.
5. Tran TP, Tu H, Pipinos II, Muelleman RL, Albadawi H, Li Y-L. Tourniquet-induced acute ischemia-reperfusion injury in mouse skeletal muscles: involvement of superoxide. Eur J Pharmacol 2011;650: 328-34.
6. Javadov S, Karmazyn M, Escobales N. Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. J Pharmacol Exp Ther 2009;330:670-8.
7. Mansour Z, Bouitbir J, Charles AL, Talha S, Kindo M, Pottecher J, et al. Remote and local ischemic preconditioning equivalently protects rat skeletal muscle mitochondrial function during experimental aortic cross-clamping. J Vasc Surg 2012;55:497-505.
8. Charles AL, Guilbert AS, Bouitbir J, Goette-Di Marco P, Enache I, Zoll J, et al. Effect of postconditioning on mitochondrial dysfunction in experimental aortic cross-clamping. Br J Surg 2011;98:511-6.
9. Mansour Z, Charles AL, Bouitbir J, Pottecher J, Kindo M, Mazzucotelli J-P, et al. Remote and local ischemic postconditioning further impaired skeletal muscle mitochondrial function after ischemia-reperfusion. J Vasc Surg 2012;56:774-82.
10. Chen HY, Navia JA, Shafique S, Kassab GS. Fluid–structure interaction in aortic cross-clamping: implications for vessel injury. J Biomech 2010;43:221-7.
11. Cour M, Gomez L, Mewton N, Ovize M, Argaud L. Postconditioning: from the bench to bedside. J Cardiovasc Pharmacol Ther 2011;16: 117-30.
12. Zorov DB, Juhaszova M, Yaniv Y, Nuss HB, Wang S, Sollott SJ. Regulation and pharmacology of the mitochondrial permeability transition pore. Cardiovasc Res 2009;83:213-25.
13. Giorgio V, Soriano ME, Basso E, Bisetto E, Lippe G, Forte MA, et al. Cyclophilin D in mitochondrial pathophysiology. Biochim Biophys Acta 2010;1797:1113-8.
14. Farivar AS, Mackinnon-Patterson BC, Barnes AD, McCourtie AS, Mulligan MS. Cyclosporine modulates the response to hypoxia-reoxygenation in pulmonary artery endothelial cells. Ann Thorac Surg 2005;79:1010-6.
15. Askar IM, Bozkurt M. Protective effects of immunosuppressants and steroids against ischemia-reperfusion injury in cremaster muscle flap at microcirculatory level. Microsurgery 2002;22:361-6.
16. Eisinger DR, Sheil AG. A comparison of the effects of cyclosporin A and standard agents on primary wound healing in the rat. Surg Gynecol Obstet 1985;160:135-8.
17. Noll E, Bouitbir J, Collange O, Zoll J, Charles AL, Thaveau F, et al. Local but not systemic capillary lactate is a reperfusion biomarker in experimental acute limb ischaemia. Eur J Vasc Endovasc Surg 2012;7:1-2.
18. Luke DR, Brunner LJ, Vadiei K. Bioavailability assessment of cyclosporine in the rat. Influence of route of administration. Drug Metab Dispos 1990;18:158-62.
19. Sullivan PG, Rabchevsky AG, Hicks RR, Gibson TR, Fletcher-Turner A, Scheff SW. Dose-response curve and optimal dosing regimen of cyclosporin A after traumatic brain injury in rats. Neuroscience 2000;101:289-95.
20. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med 2008;359:473-81.
21. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg 2002;10: 620-30.
22. Eckert P, Schnackerz K. Ischemic tolerance of human skeletal muscle. Ann Plast Surg 1991;26:77-84.
23. Dick F, Li J, Giraud M-N, Kalka C, Schmidli J, Tevaearai H. Basic control of reperfusion effectively protects against reperfusion injury in a realistic rodent model of acute limb ischemia. Circulation 2008;118: 1920-8.
24. Beyersdorf F, Schlensak C. Controlled reperfusion after acute and persistent limb ischemia. Semin Vasc Surg 2009;22:52-7.
25. Thaveau F, Zoll J, Rouyer O, Chafke N, Kretz JG, Piquard F, et al. Ischemic preconditioning specifically restores complexes I and II activities of the mitochondrial respiratory chain in ischemic skeletal muscle. J Vasc Surg 2007;46:541-7.
26. McAllister SE, Ashrafpour H, Cahoon N, Huang N, Moses MA, Neligan PC, et al. Postconditioning for salvage of ischemic skeletal muscle from reperfusion injury: efficacy and mechanism. Am J Physiol Regul Integr Comp Physiol 2008;295:R681-9.
27. Sanchez H, Bigard X, Veksler V, Mettauer B, Lampert E, Lonsdorfer J, et al. Immunosuppressive treatment affects cardiac and skeletal muscle mitochondria by the toxic effect of vehicle. J Mol Cell Cardiol 2000;32: 323-31.
28. Brown GC, Borutaite V. Inhibition of mitochondrial respiratory complex I by nitric oxide, peroxynitrite and S-nitrosothiols. Biochim Biophys Acta 2004;1658:44-9.
29. Liu Y, Fiskum G, Schubert D. Generation of reactive oxygen species by the mitochondrial electron transport chain. J Neurochem 2002;80: 780-7.
30. Zoll J, N'Guessan B, Ribera F, Lampert E, Fortin D, Veksler V, et al. Preserved response of mitochondrial function to short-term endurance training in skeletal muscle of heart transplant recipients. J Am Coll Cardiol 2003;42:126-32.
31. Sanchez H, Zoll J, Bigard X, Veksler V, Mettauer B, Lampert E, et al. Effect of cyclosporin A and its vehicle on cardiac and skeletal muscle mitochondria: relationship to efficacy of the respiratory chain. Br J Pharmacol 2001;133:781-8.
32. Piquard F, Geny B, Hardy H, Chakfe N, Mettauer B, Charloux A, et al. Lack of acute cyclosporine nephrotoxicity in late heart-transplant recipients. J Heart Lung Transplant 2000;19:507-9.
33. Csukly K, Ascah A, Matas J, Gardiner PF, Fontaine E, Burelle Y. Muscle denervation promotes opening of the permeability transition pore and increases the expression of cyclophilin D. J Physiol 2006;574: 319-27.
34. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 2005;434:652-8.
35. Sayd T, Morzel M, Chambon C, Franck M, Figwer P, Larzul C, et al. Proteome analysis of the sarcoplasmic fraction of pig semimembranosus muscle: implications on meat color development. J Agric Food Chem 2006;54:2732-7.
36. Kim HS, Raskova J, Degiannis D, Raska K. Effects of cyclosporine and rapamycin on immunoglobulin production by preactivated human B cells. Clin Exp Immunol 1994;96:508-12.
37. Nabel GJ. A transformed view of cyclosporine. Nature 1999;397: 471-2.
38. Ganote CE, Armstrong SC. Effects of CCCP-induced mitochondrial uncoupling and cyclosporin A on cell volume, cell injury and preconditioning protection of isolated rabbit cardiomyocytes. J Mol Cell Cardiol 2003;35:749-59.
39. Badalzadeh R, Mohammadi M, Najafi M, Ahmadiasl N, Farajnia S, Ebrahimi H. The additive effects of ischemic postconditioning and cyclosporine-A on nitric oxide activity and functions of diabetic myocardium injured by ischemia/reperfusion. J Cardiovasc Pharmacol Ther 2012;17:181-9.
40. Guo X-S, Li Y-Z, Liu X-H, Zhang Z-Y, Liu F-Y, Hu W- C. Calcineurin mediates the protective effect of postconditioning on skeletal muscle. Shock 2011;36:312-6.

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APPENDIX (online only)

Methods

Blood cyclosporine concentrations. The ONE-MINUTE MassTox reagent kit (Chromsystems, Munich, Germany) was used for the analysis of cyclosporine A (CsA). Whole blood concentrations of cyclosporine were qualified by high-performance liquid chromatography-electrospray mass spectrometry with an Agilent 6410 apparatus (Agilent Technologies, Santa Carla, Calif). A mixture of 50 μL of sample (control or calibrator), 25 μL of reconstituted internal standard mix containing d_{12}-cyclosporine, and 100 μL of extraction buffer was vortexed for 10 seconds and incubated for 2 minutes at room temperature. Then 250 μL of precipitated reagent was added, vortexed, and centrifuged for 5 minutes at 1500*g*.

Then, 30 μL of the supernatant was injected in the liquid chromatography coupled to tandem mass spectrometry. The mass transitions were monitored at a mass-to-charge ratio of 1220 and 1203 for cyclosporine and 1232 and 1215 for d_{12}-cyclosporine. The cycle time was 1.3 minutes injection to injection.

A standard curve was constructed by plotting the peak area ratios against the theoretical whole blood concentrations of cyclosporine. The lower limit of detection was 5 ng/mL. Concentrations were linear over the range from 25 to 2000 μg/L. The intra- and interday precision values for the concentrations were all <10.0%, and the accuracy ranged from 95% to 105% of the nominal value.

Dihydroethidium staining. To detect the presence of reactive oxygen species, mainly superoxide anion, in skeletal muscles, serial sections (10 μm thick) were cut on a cryostat microtome and thaw mounted onto glass slides. After air drying, slides were incubated for 30 minutes at 37°C with 2.5 μM dihydroethidium in phosphate-buffered saline. Dihydroethidium produces red fluorescence when oxidized to ethidium bromide by superoxide anion.[1,2] After staining, sections were rinsed, air dried, mounted in Vectashield (Vector Laboratories, Burlingame, Calif), and coverslipped. The sections were examined under an epifluorescence microscope (Nikon Eclipse E800; Nikon Instruments, Amsterdam, The Netherlands) with a 20× epifluorescence objective, and emission signal was recorded with a Zeiss 573- to 637-nm filter. We analyzed micrographs using Adobe Photoshop 6.0 (Adobe Systems, San Jose, Calif) in order to determine fluorescence intensity (arbitrary units) in regions of interest. For each animal, 20 photographs were taken in randomly determined regions of the same muscle. A region of interest of red fluorescence was determined, and the optimal sensitivity and specificity for contouring were defined so as to build a script. This script was applied to the 20 photographs, and the number of pixels in each was recorded. We excluded values above the 90th and under the 10th percentile and used the mean value.

Immunohistochemical procedure. To detect inflammation in the gastrocnemius, we used monocyte macrophage-2 antibody on serial sections (10-μm thick) of muscles.[3] Briefly, muscle sections were air dried and then fixed in paraformaldehyde 4% for 3 minutes. The sections were placed in a 2% solution of hydrogen peroxide for 5 minutes, which served to reduce endogenous or pseudo-peroxidase background staining. Primary antibody was used at predetermined optimal dilutions. A standard indirect immunoperoxidase procedure using biotinylated goat anti-rat antibody (Millipore, Billerica, Mass) and streptavidin-horseradish peroxidase (Millipore) was used to detect binding of monocyte macrophage-2. The slides were developed by diaminobenzidine substrate. Tissues were counterstained briefly with hematoxylin-eosin before mounting.

Study of cyclophilin D expression in myocardium and gastrocnemius. At the end of reperfusion, hearts and gastrocnemius muscles (n = 5 per group) were rapidly removed and frozen in liquid nitrogen. Total lysates were prepared by homogenizing tissue lysis buffer (100 μL per 10 mg of tissue) containing 1% nonyl phenoxypolyethoxylethanol, 20 mM Tris-HCl, 138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 5 mM ethylenediaminetetraacetic acid, 1 mM Na_3VO_4, 20 mM NaF, and 1 mM dithiothreitol (pH 8), supplemented with a cocktail of protease inhibitors. Protein content was evaluated using colorimetric Bradford technique in a range from 0 to 1500 μg/mL of bovine serum albumin (BSA). Thirty micrograms of heart protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on gradient gels (15% acrylamide) and transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat milk in Tris-buffered saline. Membranes then were incubated in Tris-buffered saline–Tween 20 (0.5%)–1% nonfat milk (4°C overnight) with mouse CyP D antibody (1/4000; Merck Chemicals, Nottingham, UK) or rabbit actin antibody (1/1000; Sigma Aldrich, Saint Quentin-Fallavier, France). Blots were washed and incubated with horseradish peroxidase conjugated to appropriate anti-immunoglobulin G (1/25000; Amersham GE Healthcare Europe GmbH, Saclay, France) for 1 hour at room temperature. Enhanced chemiluminescence was performed with the ECL Western blot detection kit (Amersham GE Healthcare Europe GmbH) according to the manufacturer's instructions, and blots were exposed to hyperfilm. Equal loading was evaluated by actin staining. Density of protein bands was computerized (Image J software; National Institutes of Health, Bethesda, Md).

Calcium retention capacity measurements in gastrocnemius and myocardium. Fiber bundles were first permeabilized with saponin and washed once in buffer S. Fibers then were incubated for 30 minutes with agitation at 4°C in buffer R + KCl (KCl 800 mM, CaK_2 ethylene glycol tetraacetic acid (EGTA) 2.77 mM, K_2EGTA 7.23 mM, $MgCl_2$ 1.38 mM, imidazole 20 mM, taurine 20 mM, dithiothreitol 0.5 mM, K-sulfonate of methane 90 mM, Na-sulfonate of methane 10 mM, glutamate 5 mM, malate 2 mM, K_2HPO_4 3 mM; pH 7) to extract myosin and washed three times in calcium retention capacity (CRC) + BSA buffer for 10 minutes each (Tris-base 20 mM, saccharose 150 mM, KCl

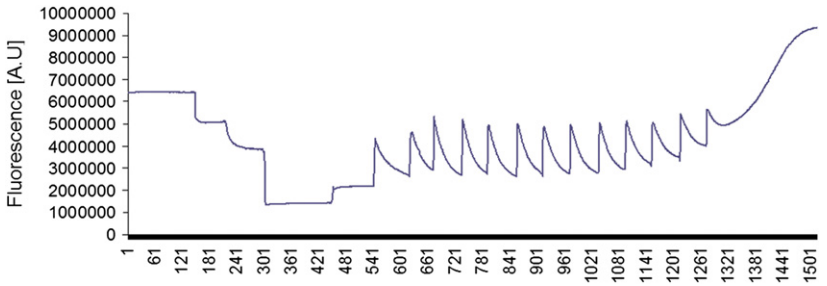

Fig 6 (online only). Typical example of Ca^{2+}-induced mitochondrial permeability transition pore (mPTP) opening recording in mitochondria of ghost fibers. *AU*, Arbitrary unit.

50 mM, KH_2PO_4 2 mM, succinate 5 mM, EGTA 9 mM, BSA 2 mg/mL; pH 7.4).

Ca^{2+} challenge. To determine sensitivity to mitochondrial permeability transition pore (mPTP) opening, we submitted mitochondria within permeabilized fibers to progressive Ca^{2+} loading of the matrix under energized conditions.[4,5] Binding of exogenous Ca^{2+} to the contractile filaments, as well as Ca^{2+} uptake by the sarcoplasmic reticulum, could interfere with measurement of mitochondrial Ca^{2+} uptake and release. To avoid these potential problems, we determined CRC in ghost fibers. For these CRC measurements, ghost fibers (1.0-1.5 mg dry fiber weight) were incubated at 24°C in a quartz tank with continuous magnetic stirring in 1 mL of CRC buffer (Tris-base 20 mM, saccharose 150 mM, KCl 50 mM, KH_2PO_4 2 mM, succinate 5 mM, EGTA 9 mM; pH 7,4). Five microliters of Ca^{2+} (1 mM) pulses were given every 5 minutes for skeletal muscle and every 2 minutes for heart fibers. Each pulse was recorded as a peak of extramitochondrial Ca^{2+} concentration using the fluorescent probe calcium green (1 μM; excitation emission, 500-530 nm). Ca^{2+} is then very rapidly taken up by the mitochondria, resulting in a return of extramitochondrial Ca^{2+} concentration to near baseline level. Following sufficient Ca^{2+} loading, extramitochondrial Ca^{2+} concentration abruptly increases, indicating a massive release of Ca^{2+} by mitochondria due to mPTP opening. A typical example of Ca^{2+}-induced mPTP opening recording in mitochondria of ghost fibers is given in Fig 6 (online only).

RESULTS

Results are given in the Table (online only).

Table (online only). Mitochondrial respiratory chain complexes' activities in sham rats treated or not treated with cyclosporine A

	Group		
Mitochondrial respiration parameter	*Untreated sham rats (n = 11)*	*CsA-treated sham rats (n = 7)*	P *value*
V_{max}	6.60 ± 0.53	6.31 ± 0.50	.72
V_{succ}	4.40 ± 0.41	4.05 ± 0.26	.56
$V_{TMPD/Asc}$	10.96 ± 0.96	9.13 ± 1.04	.24

CsA, Cyclosporine A; $V_{max,}$ maximal oxidative capacity; $V_{succ,}$ complex II, III, and IV activities; $V_{TMPD/Asc,}$ complex IV activity.

REFERENCES

1. Dikalov S, Griendling KK, Harrison DG. Measurement of reactive oxygen species in cardiovascular studies. Hypertension 2007;49: 717-27.
2. Mulsch A, Oelze M, Kloss S, Mollnau H, Topfer A, Smolenski A, et al. Effects of in vivo nitroglycerin treatment on activity and expression of the guanylyl cyclase and cGMP-dependent protein kinase and their downstream target vasodilator-stimulated phosphoprotein in aorta. Circulation 2001;103:2188-94.
3. Mansour Z, Charles AL, Bouitbir J, Pottecher J, Kindo M, Mazzucotelli JP, et al. Remote and local ischemic post-conditioning further impaired skeletal muscle mitochondrial function after ischemia-reperfusion. J Vasc Surg 2012;56:774-82.
4. Paillard M, Gomez L, Augeul L, Loufouat J, Lesnefsky EJ, Ovize M. Postconditioning inhibits mPTP opening independent of oxidative phosphorylation and membrane potential. J Mol Cell Cardiol 2009;46: 902-9.
5. Picard M, Csukly K, Robillard ME, Godin R, Ascah A, Bourcier-Lucas C, et al. Resistance to Ca^{2+}-induced opening of the permeability transition pore differs in mitochondria from glycolytic and oxidative muscles. Am J Physiol Regul Integr Comp Physiol 2008;295:R659-68.